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Knockdown Wiskott-Aldrich syndrome protein family member 3 (WASF3) inhibits colorectal cancer metastasis and sensitizes to cisplatin through targeting ZNF471

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Abstract: Colorectal cancer (CRC) is a heterogeneous cancer, and many risk factors for colorectal cancer have been established. For CRC metastasis, tumor cell migration, adhesion as well as invasion are important processes. Wiskott-Aldrich syndrome protein family member 3 (WASF3) is necessary for metastasis of various types of cancers. However, its role in CRC progression has not been fully elucidated. This study examined the *in vitro* functional roles of WASF3 in the CRC and explored the underlying molecular mechanisms. We used siRNA-WASF3 to gene silence WASF3 in colon cancer cell (HCT116) *in vitro*. The effects of WASF3 silencing on HCT116 cell apoptosis, proliferation, migration, as well as invasion were assessed by flow cytometry, CCK-8, and transwell assays. ZNF471 protein expressions were detected by immunofluorescence staining and RT-PCR. Moreover, the effects of ZNF471 were studied on a series of *in vitro* antitumor-promoting assays using HCT116. WASF3 knockdown expression using small interfering RNA (siRNA) ameliorated CRC cell proliferation, anchorage-independent growth, invasion, and metastasis. Furthermore, we observed that WASF3 contributed to upregulating the metastasis signaling pathway through inhibiting the expression of ZNF471. Our study suggests that targeting WASF3 signaling might be a novel therapeutic strategy for treating CRC.

Introduction

Due to its invasiveness and metastasis, CRC is an aggressive gastrointestinal cancer (Mendelaar *et al.*, 2021). During CRC diagnosis, around 70% of patients already have an advanced or metastatic disease (Testa *et al.*, 2020). The most common therapeutic interventions for patients with CRC include radiotherapy, chemotherapy, and surgery (Yaeger *et al.*, 2018). Chemotherapy is the main treatment option for a wide range of cancers, but CRC can develop resistance to chemotherapy (Zhang *et al.*, 2020). Evaluation of the mechanisms involved in CRC metastasis and identification of potential therapeutic targets will lead to the development of effective strategies for CRC treatment.

The Wiskott-Aldrich syndrome protein (WASp) is an actin filament nucleation protein (Liu *et al.*, 2013), Wiskott-Aldrich syndrome protein family member 3 (WASF3) is a member of the Wiskott-Aldrich family (Nie *et al.*, 2020). WASF3 participates in cell proliferation, invasion migration, and apoptosis (Nie *et al.*, 2020). WASF3 mutants can induce EMT programs to drive cancer metastasis (Wang *et al.*, 2017). Elevated WASF3 expression levels have been correlated with poor prognostic outcomes in non-small cell lung cancer (Wu *et al.*, 2014) as well as hepatocellular carcinoma patients (Loveless and Teng, 2021). Several studies have reported that WASF3 enhances CRC cell migration as well as invasion by activating epithelial-mesenchymal transition (EMT) through PI3K/ATK/Snail signaling (Teng *et al.*, 2017). In addition, WASF3 is involved in regulation of the expression levels of ZEB1, KISS1, MMP9, miRNA-200 family members, as well as those of all NF-κB activators involved in metastasis (Teng *et al.*, 2016a). However, expression levels of WASF3 in CRC patients and its connection with the pathogenesis of CRC and drug chemoresistance have not been investigated systematically.

Studies have evaluated the regulatory functions of zinc finger proteins in various human tumors (Engelken *et al.*, 2014), and some have been shown to promote disease

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development (Ni *et al.*, 2020). Zinc finger protein 471 (ZNF471) is a member of zinc finger proteins, which forms the largest transcription regulator superfamily (Sun *et al.*, 2020). Several studies have found that ZNF471 is suppressed by methylation of promoter CpG in ESCC cell lines and ZNF471 suppresses ESCC cell migration as well as invasion by EMT reversal (Sun *et al.*, 2020). The LIFR-AS1/miR-942-5p/ZNF471 axis is also involved in NSCLC invasion as well as metastasis (Wang *et al.*, 2020). However, current studies on the significance of ZNF471 in CRC are relatively scarce.

We investigated the biological functions of WASF3 and ZNF471 in HCT116 cell, especially to explore its role in growth and metastasis. We also investigated the association between ZNF471 and WASF3 in HCT116 cell and further investigated the role of WASF3 in HCT116 cell chemoresistance.

Materials and Methods

Cell line

Human CRC cell line (HCT116) was acquired from the Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China), cultured in modified high glucose DMEM medium (supplemented with 1% Streptomyces/penicillin and 10% FBS). Incubation at 37°C was done in a 5% CO_2 atmosphere.

Cell transfection

Using HCT116 as the template, 3 RNA interference target sequences (si-WASF3: GCTGAAGTGAAGAGGCTCAAA, mimics-ZNF471: AGGCAGCTAGAATTGGAATCA) were designed. Then, subsequent experiments were performed using the optimal kinetic parameter target. Transient HCT116 cell transfections with si-WASF3 (50 nM) and mimics-ZNF471 (50 nM) were conducted for 8 h using the Lipofectamine™ 3000 Transfection Reagent (ThermoFisher Scientific, L3000150).

Cell proliferation analysis

Assessment of HCT116 cell proliferation was done using the Cell Counting Kit (CCK)-8 (Beyotime, Shanghai, China). Briefly, cells were cultured in 96-well culture plates (5×10^3 cells/well) and treated with cisplatin (0–10 μmol/L) for 0, 12, 24, 48, 72 h. Then, the CCK-8 solution (10 μL) was added to every well after which incubation was done at 37°C. Measurement of absorbance was done using a 96-well plate reader (FLUOSTAR; BMG, Aylesbury, UK) at 450 nM. Assays were conducted in triplicates.

Quantitative real-time PCR

Total RNA extraction from cell lines was performed using the Trizol reagent (Invitrogen)/RNA Isolation kit (GeneJET RNA purification kit, Thermo Scientific) as instructed by the manufacturer. cDNA synthesis from the extracted RNA was done using random hexamers as well as reverse transcriptase (Superscript III, Invitrogen, Paisley, UK). The SYBR Green PCR Master mix and Light Cycler 480 SYBR I Master (Roche) were used for cDNA quantification. GAPDH was the loading control. The primer designing tools of IDTdna (http://www.idtdna.com) were used to design the primers. WASF3: forward, 5'-CAGAATTGATC-GCCTTGCTG-3', reverse, 5'-CTTCAGCCCATCCTTCTT-GTC-3'; GAPDH: forward- 5'-GGTCGGAGTCAACGG-ATTTG-3', reverse, 5'-GGAAGATGGTGATGGGATTTC-3'; ZNF471: forward, 5'-GAGATGACGAGTGAGATGAC-3', reverse, 5'-TGACTTCCCATCTGCTTCTC-3'. PCR conditions were: 94°C for 10 min, 40 cycles for 15 s at 94°C and for 1 min at 60°C. Analysis of every reaction was done using melting curves, and every curve had a single peak. The $2^{-\Delta\Delta Ct}$ method was used to calculate the gene expression levels, which were normalized to GAPDH.

Transwell migration and invasion assays

Cell migration as well as invasion were assessed by the Transwell chamber (8.0-mm pore size, polycarbonate membrane, Corning, NY). Cell cultures in the upper chamber were done in serum-free DMEM. In the lower chamber, DMEM with 10% FBS was added. Then, 24 h later, cells in the lower chamber were fixed after which cells that passed through the membrane were counted. Invading cells produce proteases that break down the Matrigel matrix (BDScience, Sparks, MD), thereby enabling invasion via membrane pores.

Caspase 3 activity ELISA kit

HCT116 cells were seeded in 96-well plates. Then, 20 μL of the caspase-3 Activity Assay Kit were pipetted into each well. After incubating for 2 h, the absorbance of plate was measured at 405 nm using ELISA plate reader to calculate percentages of survival cells.

Immunofluorescence assay

After dewaxing with water and a graded alcohol series, sodium citrate was added for antigen retrieval, and cells were incubated overnight in the presence of primary antibodies against WASF3 (Abcam, 1:1000), ZNF471 (Abcam, 1:1000), E-cadherin (Abcam, 1:1000), N-cadherin (Abcam, 1:1000) and Vimentin (Abcam, 1:1000) at 4°C. Then, cells were incubated for 30 min in the presence of secondary antibodies at room temperature after which the nuclei were stained with DAPI. Confocal microscopy (Olympus, Japan) was performed to obtain immunofluorescence (IF) images.

Statistical analysis

SPSS version 20.0 (SPSS, Inc., Chicago, IL) was used for statistical analyses. Data are shown as mean ± SD. Student's *t*-tests and 1- or 2-way analyses of variance were used for between and among group comparisons of means. $P < 0.05$ was considered statistically significant.

Results

WASF3 knockdown suppresses cell proliferation and increases cisplatin sensitivity in colorectal cancer cells

The IC50 of cisplatin was 0.76 μg/mL at 24 h. Si-WASF3 markedly inhibits the expression of WASF3 in HCT116 cells, as shown in Fig. 1A. HCT116 cells proliferation was significantly reduced after si-WASF3 transfection compared with NC-WASF3 transfection, as shown in Fig. 1B. To measure apoptosis, caspase 3 activity was assayed by the caspase 3 activity assay kit, as shown in Fig. 1C and

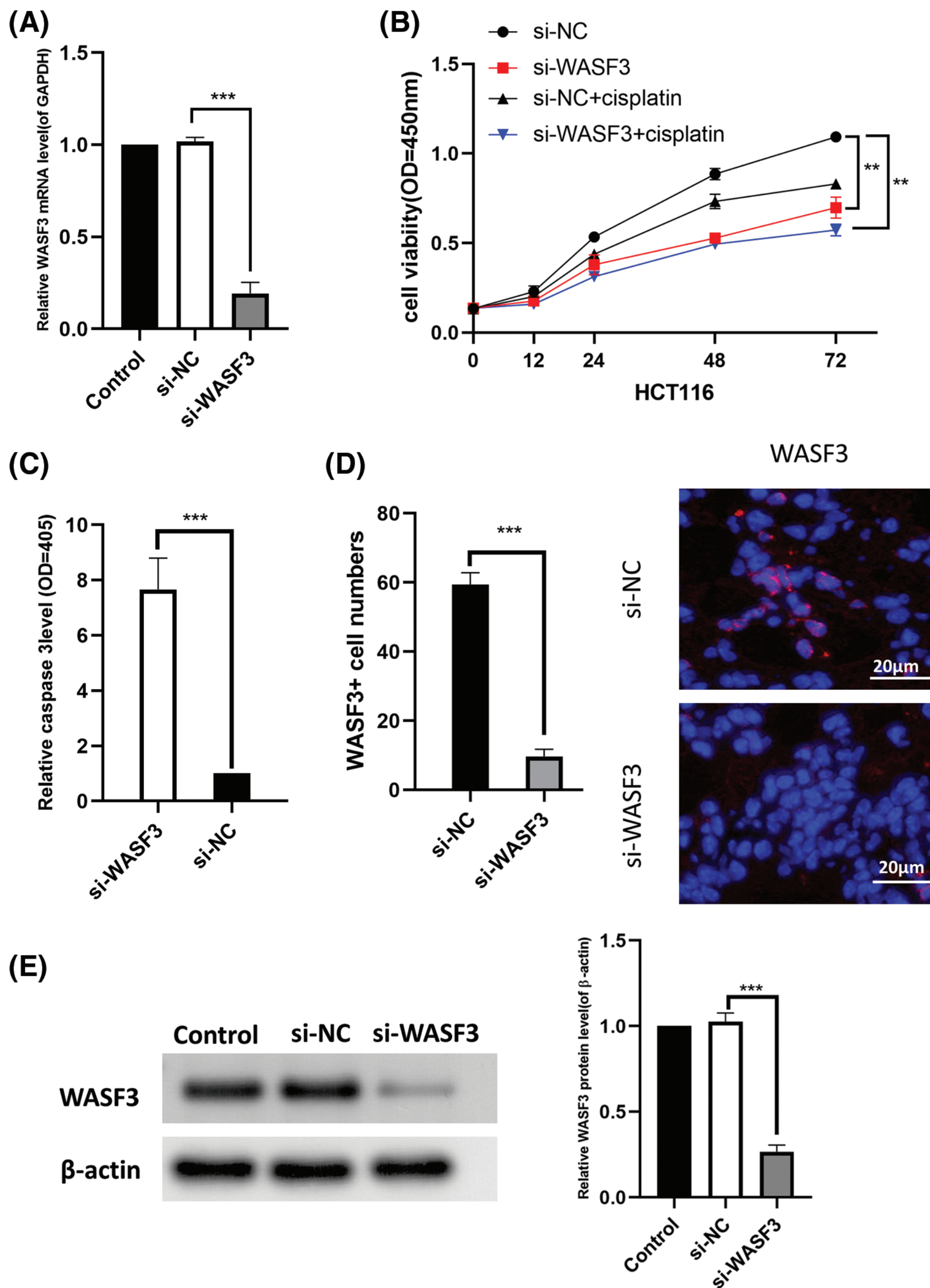

FIGURE 1. WASF3 knockdown by si-RNA inhibits HCT116 cells proliferation and sensitizes HCT116 cells to cisplatin. (A) PCR analysis of transfection efficiency of si-WASF3. (B) Proliferations in every cell group after transfections were detected by the CCK-8 kit. (C) Caspase-3 activation was assessed using the Caspase 3 Activity Assay Kit. (D) The WASF3 protein expression levels of cells after transfection were detected using immunofluorescence staining. (E) The WASF3 protein expression levels of cells after transfection were detected using western blotting (Compared with si-NC, $^{**}P < 0.01$, $^{***}P < 0.001$).

immunofluorescence staining, as shown in Fig. 1D. The data indicated that WASF3 down-regulation promoted cell apoptosis, with significant promotion of cisplatin-induced apoptosis of HCT116 cells.

Down-regulation of WASF3 inhibits migration as well as invasion of colorectal cancer cells

Migration and invasion assays revealing that WASF3 silencing significantly reduced the number of migrating, as shown in Figs. 2A and 2B and invading, as shown in Figs. 2C and 2D HCT116 cells, as well as enhanced cisplatin- inhibited metastasis.

Inhibition of WASF3 promotes colorectal cancer cell sensitivity to cisplatin and suppresses metastasis signaling pathway by targeting ZNF471

To identify potential effects of WASF3 on downstream pathways, we examined ZNF471 expression after cisplatin treatment following WASF3 gene silencing. Immunofluorescence staining results, as shown in Figs. 3A and 3B and the mRNA gene expression results obtained from RT-PCR analysis, as shown in Fig. 3C indicated that ZNF471 was upregulated with WASF3 knockdown. Note the highest level of ZNF471 expression in the cisplatin-treated si-WASF3-HCT116 cells.

Epithelial to mesenchymal transition (EMT) is a process through which epithelial cells transition to mesenchymal phenotypes directly leading to cancer cell stemness and metastasis. E-cadherin, Vimentin and N-cadherin are biomarkers of the EMT pathway. After transfection of the si-WASF3 plasmid into cells, the N-cadherin, as shown in Figs. 4A and 4B and Vimentin, as shown in Figs. 4A and 4C mRNA and protein level was downregulated and E-cadherin, as shown in Figs. 4A and 4D mRNA and protein level was upregulated, WASF3 knockdown enhanced the repressive effect of cisplatin on EMT progression.

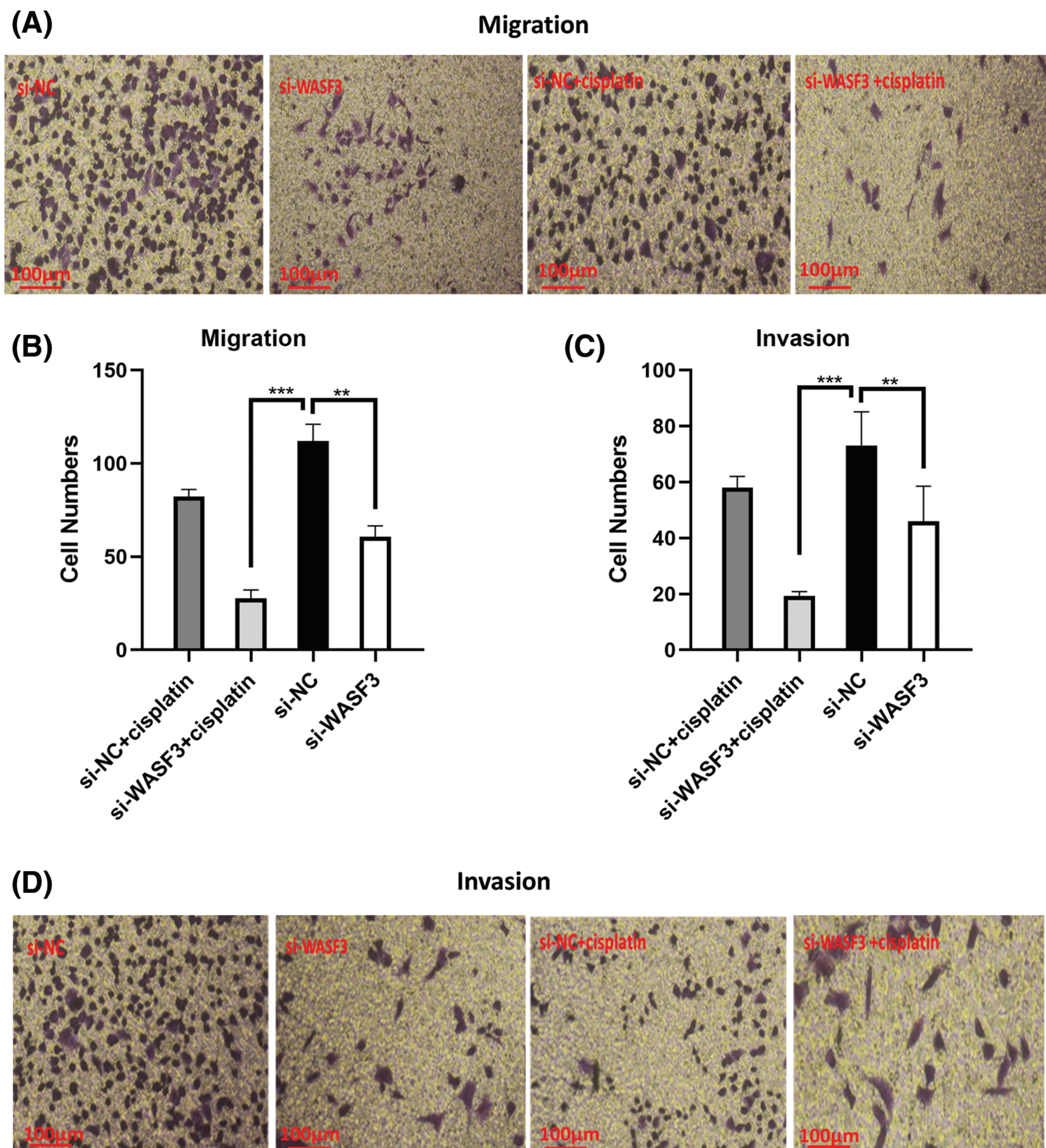

FIGURE 2. WASF3 knockdown inhibits HCT116 cell migration as well as invasion. (A–B) HCT116 cell migration was decreased after WASF3 knockdown, relative to the NC group. (C–D) The invasion of the HCT116 cells were decreased after WASF3 knockdown, relative to the NC group (Compared with si-NC, $^{**}P < 0.01$, $^{***}P < 0.001$).

ZNF471 over-expression inhibits proliferation and metastasis of colorectal cancer cells

WASF3 can promote tumorigenesis by inhibiting ZNF471 functions, then, what is the role of ZNF471 in the formation of CRC. In order to investigate it further, we overexpressed ZNF471 in HCT116 cells using miRNA mimics. Transfection efficiency was verified, as shown in Fig. 5A. CCK-8 and trans-well assays demonstrated that elevated ZNF471 expression suppressed cell proliferation, as shown in Fig. 5B, invasion, as shown in Fig. 5D, and migration, as shown in Fig. 5E. To measure the relationship between ZNF471 and apoptosis, caspase 3 activity was assayed by the caspase 3 activity assay kit, ZNF471 overexpression inhibits the expression of caspase 3, as shown in Fig. 5C. After transfection of the mimics-ZNF471 plasmid into cells, the N-cadherin and Vimentin mRNA and protein level was downregulated and E-cadherin mRNA and protein level was upregulated, as shown in Figs. 5F and 5G, ZNF471 overexpression inhibits EMT progression.

Discussion

In colon cancer patients, metastasis is a major cause of cancer-associated deaths (Liang *et al.*, 2021), therefore, it is important to elucidate on metastatic mechanisms (Pillozzi *et al.*, 2018). On the other hand, cisplatin resistance is common among CRC cancer patients with cisplatin treatment as adjuvant therapy (Teng *et al.*, 2016c). Thus, establishing the biological basis of CRC drug resistance and identifying novel targets for CRC prevention as well as treatment is vital. Moreover, elucidation of the mechanisms involved in metastatic CRC and identification of novel targets for metastasis therapy and prevention is vital.

Accumulated evidence has shown that WASF3 effectively inhibits apoptotic cell death and promotes tumor

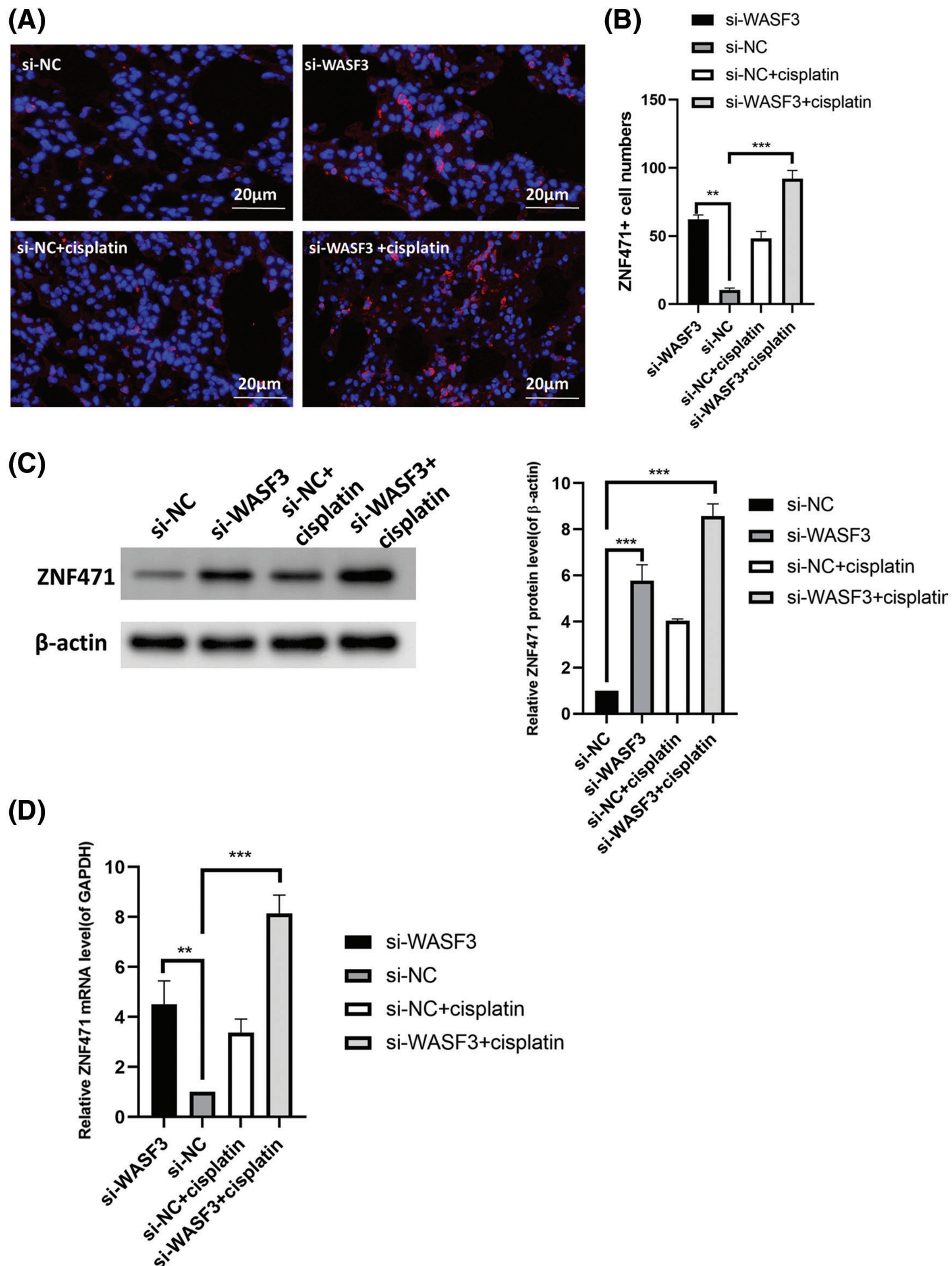

FIGURE 3. Knockdown of WASF3 promotes ZNF471 expression in CRC cells. (A–B) The ZNF471 protein expression levels of cells after si-WASF3 transfection was detected using immunofluorescence staining. (C) The ZNF471 mRNA expression levels of cells after si-WASF3 transfection were detected using western blotting. (D) The ZNF471 mRNA expression levels of cells after si-WASF3 transfection were detected using RT-PCR (Compared with si-NC, $^{**}P < 0.01$, $^{***}P < 0.001$).

proliferation, invasion, as well as metastasis in some cancer types (Teng *et al.*, 2016b). In this study we first demonstrated that WASF3 expressions were distributed in the nucleus in si-WASF3 HCT116 cells through immunofluorescence analysis and PCR. Moreover, WASF3 expression levels were positively correlated with aggressiveness of CRC. In combination, WASF3 might play a significant role in tumor tumorigenesis. Previous studies have indicated that WASF3 can induce EMT and thus enhance tumor cell migration as well as invasion (Li *et al.*, 2020). In particular, WASF3 enhances gastric cancer cell migration as well as invasion by promoting EMT via Snail upregulation (Nie *et al.*, 2020). In addition, WASF3 enhances cancer cell proliferation, migration, and invasion through the AKT pathway. WASF3 has been shown to be a

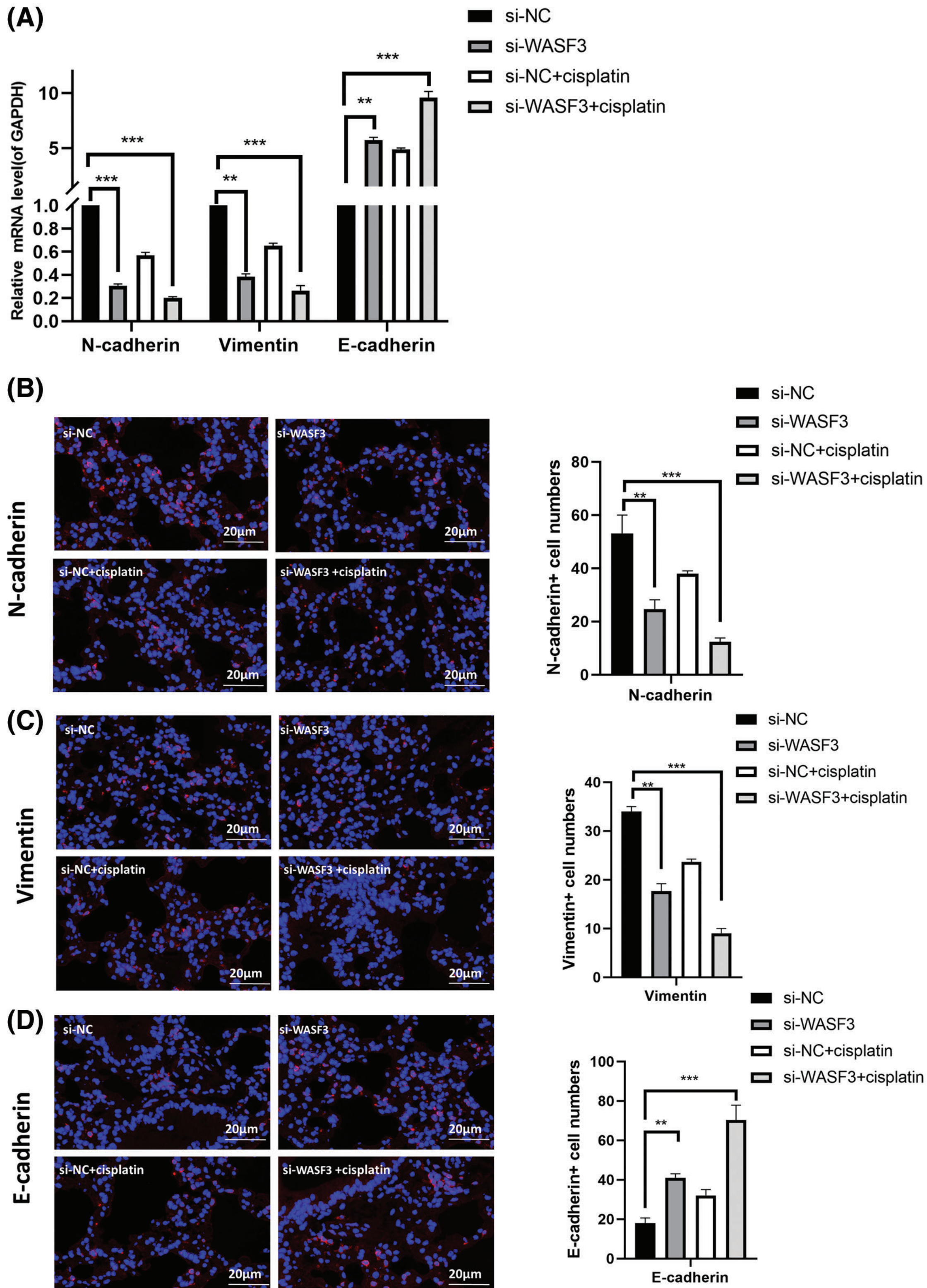

FIGURE 4. Knockdown of WASF3 inhibited EMT in CRC cells. (A) The E-cadherin, Vimentin and N-cadherin mRNA expression levels of cells after si-WASF3 transfection was detected using RT-PCR (B) The N-cadherin expression levels of cells after si-WASF3 transfection was detected using immunofluorescence staining. (C) The Vimentin expression levels of cells after si-WASF3 transfection was detected using immunofluorescence staining. (D) The E-cadherin expression levels of cells after si-WASF3 transfection was detected using immunofluorescence staining (Compared with si-NC, $^{**}P < 0.01$, $^{***}P < 0.001$).

downstream target of the anti-apoptotic PI3K signaling pathway and it controls actin polymerization as well as invasion and secretory gut epithelial cell differentiation, respectively (Teng *et al.*, 2016b).

In our study we selected HCT116 cells, which have high migration as well as invasion capacities, and WASF3 protein expression in the experiments. WASF3-knockdown caused a decrease in the migration rate of HCT116 cells suggesting the

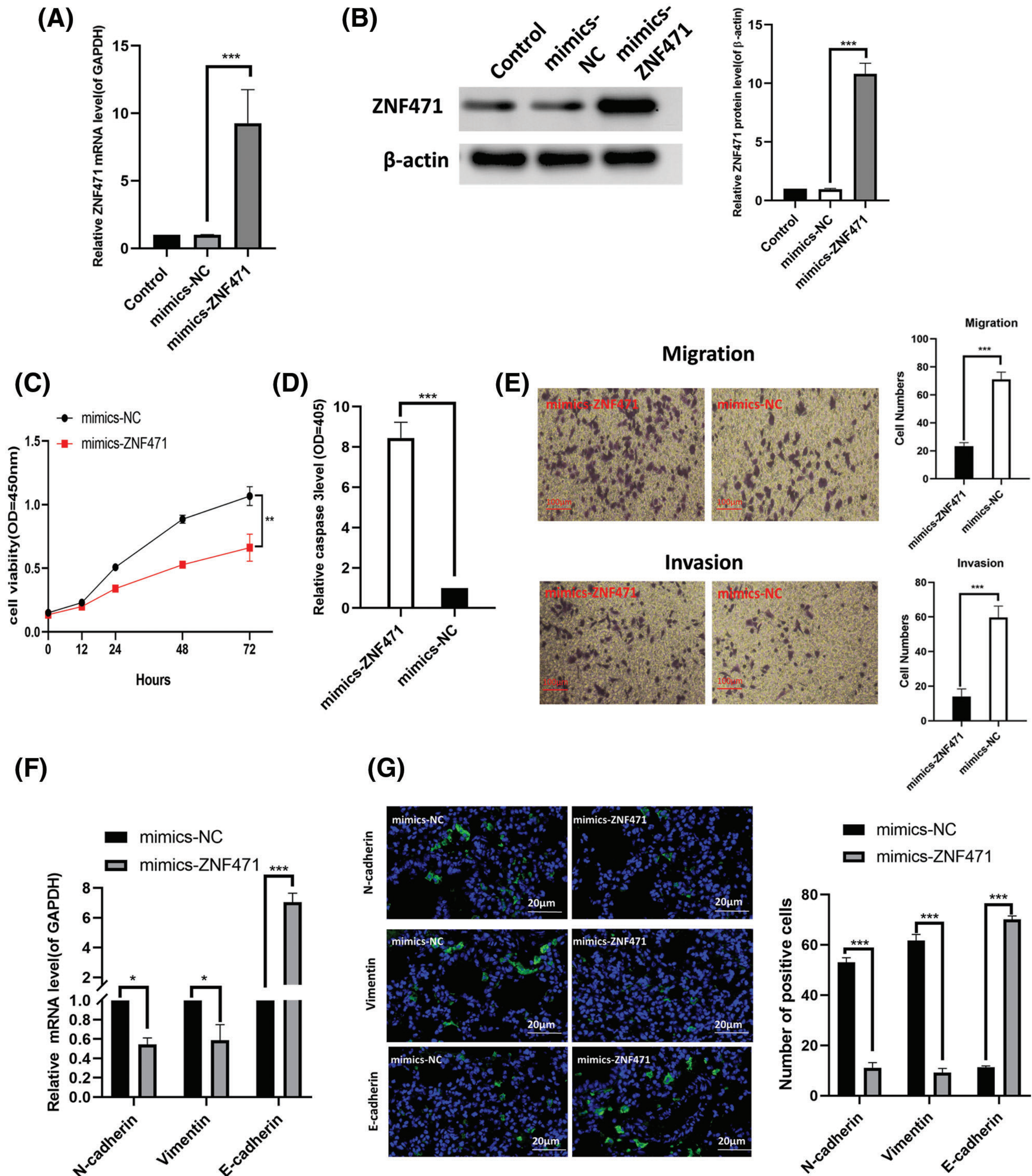

FIGURE 5. Overexpression of ZNF471 inhibits proliferation and metastasis of CRC cells. (A) PCR analysis of transfection efficiency of mimics-ZNF471. (B) Proliferation levels in every cell group after transfection were detected by the CCK-8 kit. (C) Caspase-3 activation was assessed using the Caspase 3 Activity Assay Kit in mimics-ZNF471. (D) The migration of the HCT116 cells were decreased after ZNF473 overexpression, relative to the NC group. (E) HCT116 cell invasions were decreased after ZNF473 overexpression compared to the NC group. (F) The E-cadherin, Vimentin and N-cadherin mRNA expression levels of cells after mimics-WASF3 transfection was detected using RT-PCR. (G) The EMT markers expression levels of cells after mimics-ZNF471 transfection was detected using immunofluorescence staining (Compared with mimics-NC, $^{**}P < 0.01$, $^{***}P < 0.001$).

ability of the WASF3-knockdown to lower the aggressiveness of HCT116 cells and to potentially reduce their metastatic power.

The zinc finger protein super family regulates resistance mechanisms in various biotic stresses (Guo *et al.*, 2018). Zinc Finger Protein 471 (ZNF471) is abnormally expressed in various malignancies and its abnormal expression takes part in tumor progression (Sun *et al.*, 2020). ZNF471 activity is involved in cancer initiation as well progression, but its function in tumor resistance, especially CRC, to chemotherapy is unknown. So, we investigated the role of ZNF471 in HCT116 cell. Overexpressed ZNF471 significantly suppressed HCT116 cell proliferation, migration, as well as invasion.

Moreover, we found that WASF3-knockdown increased chemosensitivity of HCT116 cells to cisplatin treatment *in vitro*, indicated by decreased cell viability and metastasis. According to our results, WASF3-knockdown induced apoptosis, metastasis and the chemosensitization of cisplatin relies on the expression of ZNF471. Thus, WASF3 is a potential prognostic as well as therapeutic target for increasing cisplatin chemosensitivity in HCT116 cell. Therefore, these results indicated that ZNF471 not only be the main target of WASF3, but also exerts tumor suppressor roles in HCT116 cell.

In conclusion, in addition to boosting cell apoptosis, WASF3 knockdown inhibited cell proliferation, migration, invasion, as well as EMT. Furthermore, WASF3-knockdown promoted cisplatin sensitivity of HCT116 cell. Notably, WASF3-induced sensitization depended on ZNF471 inhibition. Nevertheless, the effects of WASF3-induced treatment on HCT116 cell *in vivo* require further studies.

Availability of Data and Materials: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contribution: The authors confirm contribution to the paper as follows: study conception and design: Shui-Xiang He; data collection: Yan Pan, Yan Zhao; analysis and interpretation of results: Zhi-Yong Zhang, Gui-Fang Lu; draft manuscript preparation: Mu-Dan Ren, Ya-Rui Li, Yun Feng. All authors reviewed the results and approved the final version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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